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Hydrolysis of Dairy and Meat Substrates by Chicken vs. Porcine Pepsin



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Abstract.

Commercial hydrolysis requires maximizing product yields while minimizing operational costs. This article describes the optimal parameters for pepsin-catalyzed hydrolysis (enzyme origin, temperature, and pH) to achieve the highest efficiency using biologically valuable whey, casein, and meat protein substrates.

WPC-80 whey protein concentrate (79.05% protein), sodium caseinate (93.70% protein), and dry bovine heart powder (76.0% protein) served as substrates. The hydrolysis involved porcine and chicken pepsin preparations at temperatures ranging from 35 to 50°C and a pH range of 2.0–4.0. The degree of hydrolysis was spectrophotometrically assessed based on the content of TCA-soluble products. The statistical analysis relied on a two-way analysis of variance (ANOVA).

Within the pH range of 2.0–4.0, the highest yield of casein and meat protein hydrolysates occurred at pH 2.0. For the whey protein substrate, the peak yields were observed at pH 2.9 using chicken pepsin and at pH 2.3 using porcine pepsin. Increasing the temperature from 35 to 50°C consistently enhanced the yield of hydrolysis products. Although pH 2.0 corresponded to the highest initial enzyme activity, the hydrolysis of dairy proteins was less effective due to the compact native structure of whey proteins and the formation of casein aggregates at this pH.

Pepsins proved to be highly effective for hydrolyzing meat proteins as substrate accessibility increased significantly at pH 2.0. Consequently, these enzymes can be recommended for the commercial hydrolysis of denatured and aggregated meat proteins subjected to intense thermal treatment.

Keywords. Chicken pepsin, porcine pepsin, whey proteins, caseins, meat proteins, hydrolysis

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Сравнительная эффективность гидролиза куриным и свиным пепсином субстратов молочного и мясного происхождения



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Аннотация.

С помощью пепсинов могут быть получены гидролизаты, содержащие биологически активные пептиды, которые обладают антиокислительной, антигипертензивной, антимикробной и антиканцерогенной активностью. Важным аспектом производства таких гидролизатов является повышение выхода продуктов из сырья и снижение себестоимости. Цель исследования – определить параметры гидролиза пепсином (происхождение фермента, температура и pH), позволяющие достигнуть максимального выхода продуктов гидролиза на высокоценных в биологическом плане субстратах из пищевых белков (белки молочной сыворотки, казеин, мясные белки).

В качестве субстратов применяли концентрат сывороточных белков КСБ-80 (79,05 % белка), казеинат натрия (93,70 % белка) и сухой мясной порошок из сердец крупного рогатого скота (76,0 % белка). Гидролиз проводили ферментными препаратами свиного и куриного пепсина при температуре 35–50 °C и pH 2–4 ед. в соответствии с центральным композиционным планом эксперимента. Степень гидролиза оценивали спектрофотометрическим методом по содержанию продуктов гидролиза, не осаждаемых трихлоруксусной кислотой. Статистическую обработку выполняли методом двухфакторного дисперсионного анализа.

Установлено, что в исследованном диапазоне pH от 2 до 4 ед. максимальный выход продуктов гидролиза на субстратах из казеина и мясного белка наблюдался при pH 2,0 ед., для субстрата на основе нативных сывороточных белков – при pH 2,9 ед. для куриного пепсина и при pH 2,3 ед. для свиного. Повышение температуры от 35 до 50 °C увеличивало выход продуктов гидролиза. Гидролиз молочных белков пепсином при pH 2,0 ед., соответствующем максимальной активности ферментов, затруднен из-за компактной структуры сывороточных белков и образования агрегатов казеина.

Пепсины наиболее эффективны для гидролиза мясных белков, поскольку при pH 2,0 ед. их доступность для гидролиза возрастает. Пепсины рекомендуются для гидролиза мясных белков, особенно подвергшихся интенсивной тепловой обработке, сильно денатурированных и агрегированных.

Ключевые слова. Куриный пепсин, свиной пепсин, сывороточные белки, казеины, мясные белки, гидролиз

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Introduction

Pepsin (Enzym commission EC 3.4.23.1) is an acidic protease with the highest activity in the pH range of 2.0–4.0. It remains active up to 60°C [1]. This protein is rich in acidic amino acid residues (43 out of 327), resulting in a very low isoelectric point (~ pH 1.0), above which it acquires a net positive charge. Its catalytic center consists of two aspartic acid residues: Asp32 and Asp215. Pepsin activity depends on the protonation state of these residues, which, in turn, depends on the pH. The catalytic activity requires one aspartic residue

to be protonated and the other to be deprotonated. This condition corresponds with the pH range of 1.0–5.0. At pH 5.0–7.0, pepsin activity drops due to insufficient ionization of the active center. At pH ≥ 7.0, pepsin undergoes denaturation [2, 3].

The pH level also affects the electrostatic interaction forces between the pepsin and the protein substrate. In a strongly acidic environment, where pH approximates 1.5–2.5, pepsin carries a net negative charge, whereas food proteins typically carry a net positive charge. These opposite charges lead to electrostatic attraction

between the enzyme and the substrate, thereby boosting hydrolysis [4].

The pH profile of pepsin activity varies depending on the substrate type and its three-dimensional structure. Studies using serum albumin as a substrate show that the activity of human pepsin, like that of porcine pepsin, peaks at pH 2.0 and falls down to a negligible level at pH ≥ 5.0 [5]. In an experiment with native hemoglobin, porcine pepsin activity was reported to peak at a pH of approximately 2.0 and declined as the pH rose to 4.0 [3]. A similar pattern for porcine pepsin was observed with denatured hemoglobin, whereas chicken pepsin exhibited peak activity at pH 2.5–3.0. During the hydrolysis of plant-derived (wheat, peas) proteins, the highest activity for both porcine and chicken pepsin occurred at pH 1.5 [6]. Pepsin activity against native egg albumin and bovine serum albumin peaked at pH ~ 2.0 , shifting to higher pH values (3.0 and 4.0, respectively) when these proteins were in a denatured state. The optimal pH for pepsin activity against urea-denatured hemoglobin shifted from 2.0 to ~ 3.5 , with activity maintained up to pH 5.0 [5].

Pepsin cleaves peptide bonds between specific amino acid residues. Since its active site contains hydrophobic regions, it preferentially cleaves bonds formed by hydrophobic amino acids, including phenylalanine, tyrosine, tryptophan, leucine, methionine, and isoleucine [3, 7]. This specificity is pH-dependent. In the hydrolysis of myofibrillar proteins, the highest specificity was recorded at pH ~ 1.3 , where the enzyme cleaves only those bonds that involve phenylalanine and leucine. At pH ≥ 2.0 , the number of cleavable bond variants increases [3].

Pepsin is used in collagen production [8] and leather processing [9]. The pharmaceutical industry produces a pepsin preparation, which is prescribed as an auxiliary digestive agent [10]. The food industry uses pepsin in cheese production [11], while enzymatic modifications with pepsin are employed to tenderize meat and produce ready-to-eat foods, particularly meat sauces [12]. Pepsin is also utilized to produce enzymatic hydrolysates of animal and plant-derived food proteins with varying degrees of hydrolysis and functional activity [13–15].

Peptic hydrolysis of proteins is an essential stage in the digestion of food. Peptic protein hydrolysates serve as protein supplements for senior population, who secrete less pepsin with age [16]. Hydrolysis recovers food-grade protein from meat and fish industry waste [17, 18]. This approach enables the production of additional protein products from low-cost raw materials while reducing the volume of environmentally polluting waste. To separate meat proteins from non-hydrolyzable raw material components (bones, keratin impurities, etc.), the material undergoes preliminary processing using various techniques, including heating, high-pressure treatment, exposure to electric fields, or various combined treatments. The resulting meat substrates (suspensions, broths, or pastes) are free from ballast substances [11–20].

However, extensive denaturation and aggregation of meat proteins hinder subsequent hydrolysis [21]. Despite a lower hydrolysis rate, pepsin effectively hydrolyzes meat raw materials composed of aggregated proteins [22]. In addition, pepsin-derived products contain bioactive peptides that are beneficial for human physiology [23–25].

Pepsin owes its ability to generate bioactive peptides to its selectivity. This enzyme specifically targets the cleavage of bonds involving hydrophobic amino acids. Consequently, the peptides released during peptic hydrolysis feature hydrophobic amino acids at their termini [7]. Such peptides exhibit greater biological activity compared to peptides lacking hydrophobic terminal groups [23]. Peptic hydrolysates demonstrate the following activity types, depending on the type of protein substrate and the hydrolysis conditions:

- antioxidant activity (they protect body cells against damage by neutralizing peroxides and free radicals [26, 27]);
- they inhibit angiotensin-converting enzyme and dipeptidyl peptidase IV, thus reducing blood pressure and facilitating the treatment of type 2 diabetes [28];
- antihypertensive properties (they lower blood pressure [29]);
- antimicrobial activity (they inhibit the viability of pathogenic microorganisms [30]);
- anticarcinogenic activity (some peptides induce cancer cell death [31]).

Pepsin can also yield products with synergetic biological and functional properties, e.g., low antigenicity [32], reduced allergenicity, antioxidant, anticarcinogenic, and immunomodulatory effects, etc. [33].

The economic aspect of hydrolysate production is as important as the production of hydrolysates with targeted functional properties. Optimal pepsin hydrolysis conditions reduce processing time and increase the hydrolysate yield while reducing the raw material, labor, and energy costs. This study explores the pepsin hydrolysis parameters (enzyme source, temperature, and pH) that maximize the yield of hydrolysis products from biologically valuable food protein substrates (whey proteins, casein, and meat proteins).

Study objects and methods

Native whey protein concentrate (WPC-80) served as the source of whey proteins. It was obtained via ultrafiltration and contained 79.05% protein (Molochny Mir, Belarus).

Sodium caseinate with a protein content of 93.70% was prepared by skimming raw cow's milk (Agrivolga, Russia) in a cream separator to obtain a fat content of 0.05%. The milk was placed in a jacketed vessel equipped with an agitator, cooled down to $8 \pm 2^\circ\text{C}$, and stirred while adding a 10% hydrochloric acid solution until the pH reached 4.5 ± 0.1 . The acidified mix was heated to $50 \pm 1^\circ\text{C}$ while stirring and allowed to settle to separate the casein precipitate. After that, we washed the precipitate to remove acid, lactose, and soluble minerals,

removed the supernatant by siphoning, and added deionized water to the precipitate ($15 \pm 5^\circ\text{C}$) in a volume equal to half the initial volume of skim milk.

The acidity of the mix was adjusted to $\text{pH } 4.7 \pm 0.1$ by adding a 10% hydrochloric acid solution, after which the precipitate settled for 16–18 h. The process included seven washing cycles. After the final cycle, the mix went through polyester fabric, and the casein precipitate was placed in a cheese mold for 2 h to drain moisture.

The drained casein precipitate was transferred to a vessel equipped with a stirrer, and deionized water was added in an amount equal to half the initial volume of the skimmed milk. To dissolve casein, the pH of the solution was adjusted to 6.6 ± 0.1 by adding a 20% (w/w) NaOH solution under continuous stirring.

The neutralized mix was dried in a Niro-Atomizer spray dryer (Niro A/S, Denmark) with air temperatures of $150 \pm 2^\circ\text{C}$ at the inlet and $80 \pm 2^\circ\text{C}$ at the outlet of the drying chamber.

Dry meat powder with a protein mass fraction of 76.0% was produced from chilled bovine hearts with a protein mass fraction of 17.15% (Agrivolga, Russia). After grinding the raw material in a meat grinder, we sterilized the mince in an HC-O002 autoclave (Guangzhou Happycare Electronics, China) at $121 \pm 1^\circ\text{C}$ for 20 min. After cooling, it was dispersed using a Biolo-Mix A8800 blender (BioloMix Trading, China). The dispersed mince was dried in a forced-air drying oven at 55°C for 4 h on silicone trays, ground in a coffee grinder, and sieved through a 24-mesh screen to remove particles that exceeded 0.7 mm.

The pepsin enzyme preparations included porcine pepsin with a proteolytic activity of 5,734 proteolytic units/g (SHAKO, Russia) and chicken pepsin with a proteolytic activity of 5,423 proteolytic units/g (Endocrine Enzyme Plant, Russia).

The protein mass fraction was determined in milk proteins and milk protein hydrolysates according to State Standard GOST 23327-98. For meat proteins and meat protein hydrolysates, the protocol followed State Standard GOST 25011-2017.

The protein mass fraction was calculated by multiplying the nitrogen mass fraction by a conversion factor of 6.38 (for whey proteins and casein) or 6.25 (for meat proteins). The total proteolytic activity (in proteolytic units/g) was determined using the Anson method with a hemoglobin substrate, modified as in GOST 34430-2018 for acidic proteases ($\text{pH } 3.0$, 30°C).

We prepared the protein substrate solutions by mixing a weighed amount of protein raw material with water in the ratio required to obtain a solution with 2% protein mass fraction. The meat powder solution underwent additional homogenization in an ULTRA-TURRAX T 18 Digital disperser (IKA, Germany) at 15,000 rpm for 1.0–1.5 min.

To prepare protein substrate solutions with the targeted pH, we used buffer solutions based on citrate

buffer ($\text{pH } 3.0$ and 4.0) or citric acid ($\text{pH } 2.0$) at a concentration of 0.2 mol/dm^3 , following the method developed by V. S. Stoll *et al.* [34]. The substrate solutions with a protein mass fraction of 1% in a 0.1 mol/dm^3 buffer were prepared by mixing a substrate solution with a protein mass fraction of 2% and a 0.2 mol/dm^3 buffer solution in a 1:1 ratio. For better protein hydration, the resulting substrate solutions remained in a refrigerator at 4°C for 16–18 h.

After placing a 43 cm^3 volume of the substrate solutions into 50 cm^3 Falcon-type plastic tubes, we brought them to the hydrolysis temperature in a water bath. The hydrolysis was performed using an MTH-100 thermoshaker (Hangzhou Miu Instruments, China) at a shaking speed of 300 rpm (3 mm orbital diameter).

To initiate hydrolysis, we added 0.8 cm^3 of enzyme solution to the tubes containing 40 cm^3 of substrate upon removing 3 cm^3 for a blank measurement. The enzyme concentration was adjusted to achieve an enzyme-to-substrate ratio of 40 proteolytic units/g of protein. The enzyme entered each successive tube at intervals of $30 \pm 2 \text{ s}$. The hydrolysis lasted 15 min for the casein-based substrate and 90 min for the substrates based on whey or meat proteins. The samples left the test tubes in the same sequence at intervals of $30 \pm 2 \text{ s}$.

The enzyme dose and hydrolysis time were determined preliminarily to yield a hydrolysis product concentration with absorbance values within the 0.1–1.0 range as determined by spectrophotometry.

The content of hydrolysis products was studied spectrophotometrically as in [3], with modified sample dilution and filtration.

Once the hydrolysis temperature was reached in the shaker tubes, a 3.0 cm^3 aliquot of substrate was withdrawn from each tube to determine the blank level. These samples were transferred to 50 cm^3 plastic Falcon-type centrifuge tubes with 9 cm^3 of trichloroacetic acid (TCA) solution. The TCA concentration was 6.7% for the casein and the meat protein substrates (final TCA concentration = 5%) and 16.1% for the whey protein substrates (final TCA concentration = 12%).

The resulting mix was vortexed in a VX-MT vortex mixer (Xiamen Branch Tongxin Biomedical Technology, China) and incubated for 14–16 h in a refrigerator at $4\text{--}5^\circ\text{C}$ to aggregate the non-hydrolyzed protein. Subsequently, the mix was centrifuged at 10,000 g for 10 min in a TsLn-16 centrifuge (Changsha Xiangzhi, China). The supernatant went through a nylon filter with $0.45 \mu\text{m}$ pores (Tianjin Jinteng Experiment Equipment, China).

The hydrolysis products included peptides and free amino acids not precipitated in the TCA solution. Their content in the filtrate was determined by measuring the optical density at 280 nm and after subtracting the optical density at 320 nm. The measurements involved a PB 2201 spectrophotometer (Spectroscopy, Optics and Lasers Advanced Developments, Belarus) equipped with quartz cuvettes with a 10 mm optical path length. To compen-

sate for the effect of substrate background absorption, we subtracted the absorbance value of the blank from the obtained values.

The microscopy of the protein substrates involved “mounted drop” preparations and transmitted-light microscopy with a dark-field condenser. The images, made with a Canon EOS 600D digital camera, were processed with Digital Photo Professional v.4.5 (Canon, Japan). Before applying them onto the microscope slide, we preheated the substrate solutions to the hydrolysis temperature and allowed them to incubate at that temperature for 30 min.

The experiment followed a central composite design for response surface methodology [35]. The experimental design involved two quantitative factors, temperature (35, 40, 45, and 50°C) and pH (2.0, 3.0, and 4.0). One series of experiments featured chicken pepsin and the other used porcine pepsin. The experiments were carried out over two days, with two replicates performed each day ($n = 4$).

The magnitude of the factors’ influence on the response variable was assessed using two-way analysis of variance (ANOVA) based on three statistical criteria: the ANOVA effect estimates, the Fisher’s F -ratio (F), and the computed significance probability (p) [35]. The factor effect represents the magnitude of the change in the response variable as the influencing factor shifts from its lower to its upper level. However, an assessment based on the factor effect is tied to the absolute value of the response variable, making it impossible to compare the strength of the factors’ influence on the response variable (hydrolysis product yield) across the different substrates and enzymes. Therefore, the F -test served as the evaluation criterion. This test reflects the impact of the factor’s variation on the variation of the response variable without depending on the absolute values of the response variable itself. The F -test provides comparable estimates of the strength of factor influence during the hydrolysis of different substrates by pepsins of various origins.

The Fisher’s F -test is designed to assess the statistical significance of the obtained result. However, under certain conditions, in particular when, within the chosen model, the factors have the same number of degrees of freedom and the residual error of the model is small (which is evidenced by a high value of the coefficient of determination R^2), the value of the F -test will reflect not only statistical significance but also the strength of the factor’s influence on the response variable. A higher F -value for a factor will indicate a greater degree of influence of the variation of this factor on the variation of the dependent variable. The Fisher’s criterion is limited, as it yields an estimate that does not account for the change vector (i.e., whether it increases or decreases) in the response variable resulting from the variation of the influencing factor. To address this issue, we indicated the sign of the factor effect in the ANOVA table.

To confirm the statistical significance of the factor’s impact on the response variable, we also included the p -value in the table. A factor’s effect was statistically significant at $p < 0.05$. The statistical analysis relied on the Statistica software (StatSoft, USA).

Results and discussion

The analysis of variance made it possible to evaluate the effect of the experimental factors on the response variable, i.e., hydrolysis yield. A model that incorporated linear and quadratic effects described the relationship between the response variable and the experimental factors, as well as the pairwise interaction effects between linear terms. Table 1 summarizes the data on the effect of each factor on the response variable.

The following regression relationships describe the dependence of the response variable (hydrolysis yield) on the factors (temperature and pH):

$$Y = a_1 \times \text{Temp} + a_2 \times \text{Temp}^2 + a_3 \times \text{pH} + a_4 \times \text{pH}^2 + a_5 \times \text{Temp} \times \text{pH} + b$$

where Y is the yield of hydrolysis products (optical density units); Temp denoted the temperature factor level (°C); pH was the pH factor level; a_i ($i = 1 \dots 5$) denoted the coefficients for the equation factors; b stood for the equation constant.

We derived regression equations from the hydrolysis of protein substrates by chicken and porcine pepsins to calculate the temperature and pH values for the highest hydrolysis yield (Table 2), as well as the plots showing the effect of temperature and pH on the response variable (Fig. 1).

The model for the relationship between the response variable and the experimental factors incorporated linear and quadratic effects, as well as pairwise interaction effects. It exhibited a coefficient of determination in the range of $0.95 < R^2 < 0.99$; that is, it accounted for 95–99% of variation in the response variable.

The linear temperature factor (Temp^L) had a strong effect on hydrolysis yield across all the substrates. Based on the positive vector sign for this effect, raising the temperature from 35 to 50°C increased the yield of hydrolysis products. The F -test values for Temp^L showed that the chicken and the porcine pepsins responded differently to the changes in temperature and pH. When acting on the same substrate, a temperature change of 35–50°C had a greater impact on the hydrolysis yield for chicken pepsin than for porcine pepsin. Obviously, chicken pepsin was more temperature-sensitive, with its activity dropping together with the temperature. The porcine pepsin activity was less dependent on temperature fluctuations within the 35–50°C range.

The pH factor (pH^L) had a strong linear effect on all substrate-pepsin combinations except for the hydrolysis of whey proteins by chicken pepsin. Based on the negative vector sign, increasing the pH from 2.0 to 4.0

resulted in a lower yield of peptic hydrolysis products. This result was in line with other studies: regardless of substrate, pepsin activity decreases as the pH rises within the 2.0–4.0 range [19]. The F -test values for pH^L indicated that the pH level had a greater effect on the activity of porcine pepsin than on that of chicken pepsin. The increase in active acidity from pH 2.0 to 4.0 corresponded to a greater loss in activity in porcine pepsin compared to chicken pepsin.

The analysis of variance indicated that the effect of factors on the response variable depended on the specific substrate-enzyme combination. This effect is

obvious from the differing estimates for the quadratic effects of temperature (Temp^Q) and pH (pH^Q), as well as from the pairwise interaction effect between the factors ($\text{Temp}^L \times \text{pH}^L$).

The hydrolysis of the whey protein substrate demonstrated the following patterns. The whey protein hydrolysis yield had a linear dependence on the hydrolysis temperature, as the quadratic effect of the temperature factor (Temp^Q) was statistically insignificant. For the pH factor, the quadratic effect (pH^Q) was statistically significant and negative. Within the pH range of 2.0 to 4.0, the dependence of the response variable on the

Table 1. Effect of experimental factors on hydrolysis yield: Dispersion analysis

Таблица 1. Оценка влияния факторов эксперимента на выход продуктов гидролиза по результатам дисперсионного анализа

Factor	Substrate	Enzyme					
		Chicken pepsin			Porcine pepsin		
		Effect sign	F -ratio	p -value	Effect sign	F -ratio	p -value
Hydrolysis temperature ^L (Temp^L)	Whey protein	+	370.05	***	+	262.79	***
	Casein	+	678.00	***	+	333.01	***
	Meat proteins	+	476.05	***	+	210.54	***
Hydrolysis temperature ^Q (Temp^Q)	Whey protein	+	3.50	–	+	2.92	–
	Casein	–	30.18	***	–	1.86	–
	Meat proteins	+	4.54	*	+	0.55	–
pH^L	Whey protein	–	2.64	–	–	573.18	***
	Casein	–	695.70	***	–	1,580.47	***
	Meat proteins	–	1,362.07	***	–	2,621.21	***
pH^Q	Whey protein	–	446.32	***	–	185.24	***
	Casein	–	8.35	**	+	23.77	***
	Meat proteins	+	34.72	***	+	323.85	***
$\text{Temp}^L \times \text{pH}^L$	Whey protein	–	6.46	*	–	33.93	***
	Casein	+	20.63	***	+	44.40	***
	Meat proteins	–	77.49	***	–	135.66	***
R^2	Whey protein	0.95			0.96		
	Casein	0.97			0.98		
	Meat proteins	0.98			0.99		

Note: ^L – linear effect; ^Q – quadratic effect; R^2 – multiple coefficient of determination; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Dash (–) indicates not statistically significant effect ($p > 0.05$).

Примечание: ^L – оценка линейного эффекта фактора; ^Q – оценка квадратичного эффекта фактора; R^2 – коэффициент детерминации аппроксимирующей модели; * – $p < 0,05$; ** – $p < 0,01$; *** – $p < 0,001$. «–» – значение статистически недостоверно ($p > 0,05$).

Table 2. Temperature and pH for the highest hydrolysis yield across the protein substrates

Таблица 2. Значения температуры и pH, соответствующие максимальному выходу продуктов гидролиза исследуемых белковых субстратов

Factor	Substrate	Enzyme	
		Chicken pepsin	Porcine pepsin
Temperature, °C	Whey protein	50.0	50.0
	Casein	50.0	50.0
	Meat proteins	50.0	50.0
pH	Whey protein	2.9	2.3
	Casein	2.0	2.0
	Meat proteins	2.0	2.0

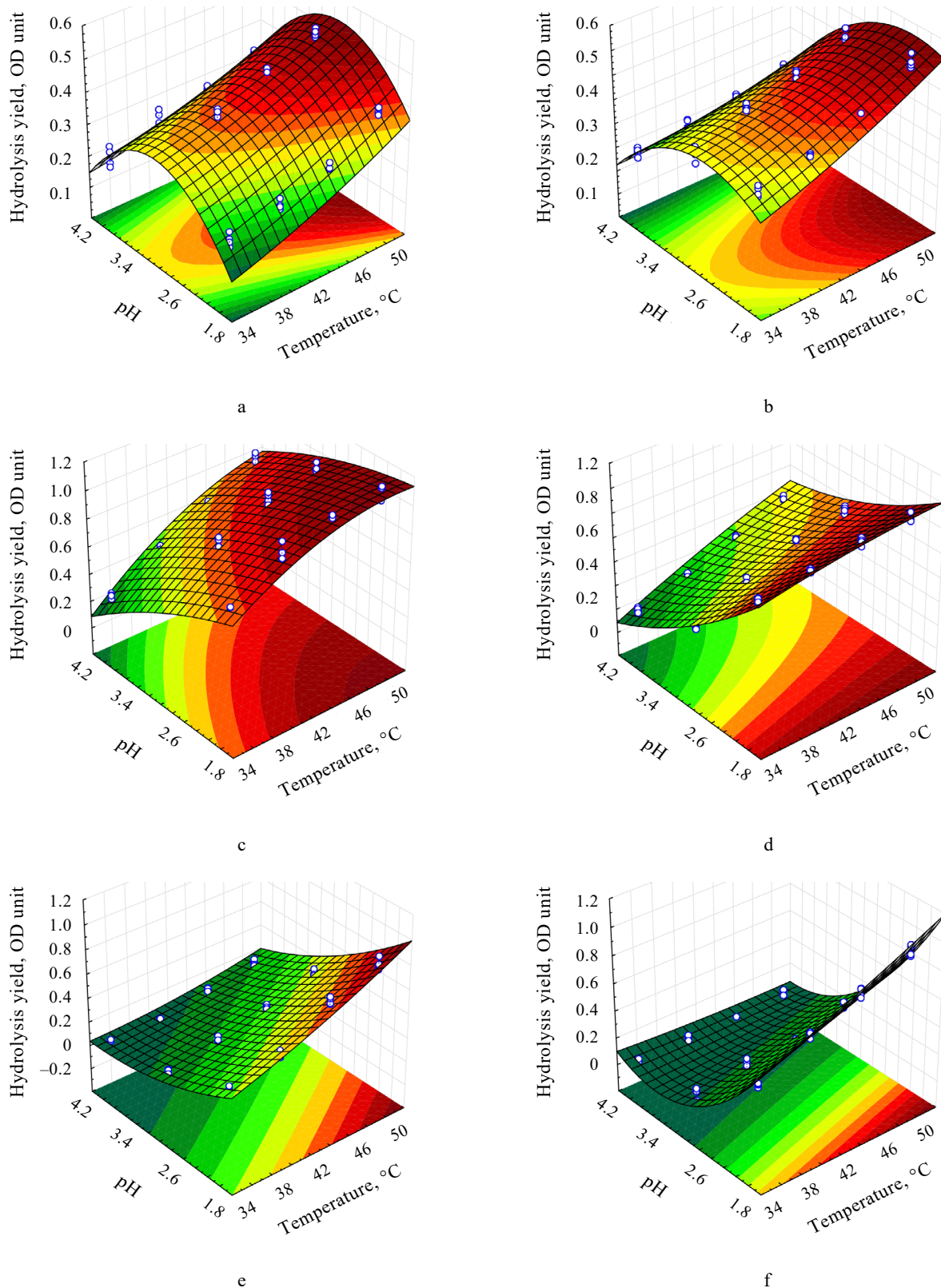


Figure 1. Effect of temperature and pH on yield surfaces: a – whey protein and chicken pepsin; b – whey protein and porcine pepsin; c – casein and chicken pepsin; d – casein and porcine pepsin; e – meat protein and chicken pepsin; and f – meat protein and porcine pepsin

Рисунок 1. Поверхности отклика выхода продуктов гидролиза в зависимости от температуры и pH при гидролизе белковых субстратов: а – сывороточный белок и куриный пепсин; б – сывороточный белок и свиной пепсин; в – казеин и куриный пепсин; д – казеин и свиной пепсин; е – мясной белок и куриный пепсин; ф – мясной белок и свиной пепсин

pH followed an inverted parabola shape that peaked at the vertex. As pH increased, the hydrolysis product yield initially rose and then fell, peaking at a specific pH value.

For chicken pepsin, the linear effect of the pH factor (pH^{L}) was not significant ($p > 0.05$) for the response variable. The values at the extremes of the factor's range (pH 2.0 and 4.0) demonstrated no statistical significance either. For porcine pepsin, both the linear (pH^{L}) and quadratic (pH^{Q}) effects of the pH factor significantly affected the response variable, with both effects being negative.

As with chicken pepsin, the dependence of the response variable on pH followed an inverted parabola shape, but the values at the range extremes differed: the response variable value was lower at the upper pH level (pH 4.0) than at the lower one (pH 2.0). A statistically significant pairwise interaction effect occurred between temperature and pH ($\text{Temp}^{\text{L}} \times \text{pH}^{\text{L}}$). Although small in absolute magnitude, the effect was negative. Therefore, these factors had a mutually reinforcing effect, where the highest hydrolysis yield was achieved by higher temperatures at lower pH values.

Pepsin activity was earlier reported to peak at pH 2.0. As the pH rose from 2.0 to 4.0, the pepsin activity decreased. However, the pH level affected not only enzyme activity but also substrate accessibility for hydrolysis. It was the protein's spatial structure that served as the primary factor affecting the accessibility of whey proteins to enzymatic hydrolysis (by pepsin). β -Lactoglobulin became resistant to pepsin hydrolysis at low pH levels (≤ 3.0), as strengthened intramolecular hydrogen bonds compacted the protein into a tight structure [36, 37]. Consequently, most potential pepsin cleavage sites with hydrophobic amino acids became sequestered within the hydrophobic core of the β -lactoglobulin molecule, making them largely inaccessible to the enzyme [36, 38].

The hydrolysis of β -lactoglobulin was reported to release peptides that exhibited a high propensity to aggregate via intermolecular β -sheets at pH 2.0. This aggregation tendency was typical of specific peptides, primarily those with hydrophobic regions, a low net charge (at pH 2.0), and no proline residues [39].

The susceptibility of α -lactalbumin to pepsin hydrolysis is also known to depend on the protein's spatial structure. Unlike β -lactoglobulin, the tertiary structure of α -lactalbumin does not become more compact when the pH drops below 3.0. Instead, it completely unfolds, exposing all its polypeptide bonds to the enzyme. When the pH rises to 4.0, α -lactalbumin regains a compact three-dimensional structure that hinders enzyme access to potential hydrolysis sites [1].

According to A. Ye *et al.* [37], α -lactalbumin and bovine serum albumin were hydrolyzed by pepsin significantly faster than β -lactoglobulin at the same pH level. As reported by K. L. Jakopović *et al.* [1], β -lactoglobulin underwent virtually no hydrolysis by pepsin at the pH

range of 2.5–3.5, whereas α -lactalbumin actively participated in peptic hydrolysis. After 60 min of hydrolysis at 40–45°C, 95–100% of β -lactoglobulin remained unhydrolyzed, compared to $\leq 20\%$ in α -lactalbumin.

The susceptibility of whey proteins to enzymatic hydrolysis also depends on their degree of aggregation [40]. Since the production of whey protein concentrate involves a heat treatment step, i.e., pasteurization, a portion of the constituent proteins denatures and becomes able to form aggregates at $\text{pH} < 4.0$ [41].

We used light microscopy to study the formation of whey protein aggregates in the substrates. Figure 2 shows micrographs of protein substrates based on whey proteins in buffer solutions at pH 2.0, 3.0, and 4.0.

Prior to hydrolysis, whey protein solutions contained aggregates whose size depended on the pH. At pH 2.0, whey proteins had a high positive charge, resulting in strong electrostatic repulsion between protein particles and preventing their association into aggregates [36]. As the pH rose from 2.0 to 4.0, the charge on the protein particles decreased, as did the electrostatic repulsion. Particle aggregation (turbidity) manifested at pH 3.0, while weak, loose protein aggregates formed in the solution at pH 4.0.

Following hydrolysis, larger and denser aggregates appeared in the whey protein solutions. The formation of post-hydrolysis aggregates was linked to the unfolding of the whey protein structure following limited hydrolysis. This process exposed hydrophobic regions and free thiol groups. The interaction between these groups drove the association of protein molecules into aggregates [42]. The size of hydrolyzed protein aggregates, like that of non-hydrolyzed protein aggregates, depended on the pH level. As the pH approached 4.0, the surface charge decreased, as did the associated electrostatic repulsion forces, promoting the formation of larger aggregates (Fig. 2). The aggregation of protein particles reduced the rate of hydrolysis because a significant proportion of the bonds susceptible to pepsin cleavage became buried within the protein aggregates. The hydrolysis rate decreased as the size and density of the aggregates increased [43].

The highest yield of whey protein hydrolysis at pH 2.0–3.0 occurred because two processes with opposing effects on hydrolysis happened simultaneously as the pH rose from 2.0 to 3.0. On one hand, the pepsin activity went down. On the other hand, the conformational changes increased the susceptibility of the major whey protein, i.e., β -lactoglobulin, to hydrolysis. These opposing factors came to balance at pH 2.0–3.0. As pH grew from 3.0 to 4.0, the aggregation of protein particles intensified, including those destabilized by hydrolysis, and inhibited the enzymatic process. Superimposed on this is the difference in the pH-dependent activity between porcine and chicken pepsins, a phenomenon documented by I. Crévieu-Gabriel *et al.* [6]. Resulting from the synergetic effect of these factors, the activity

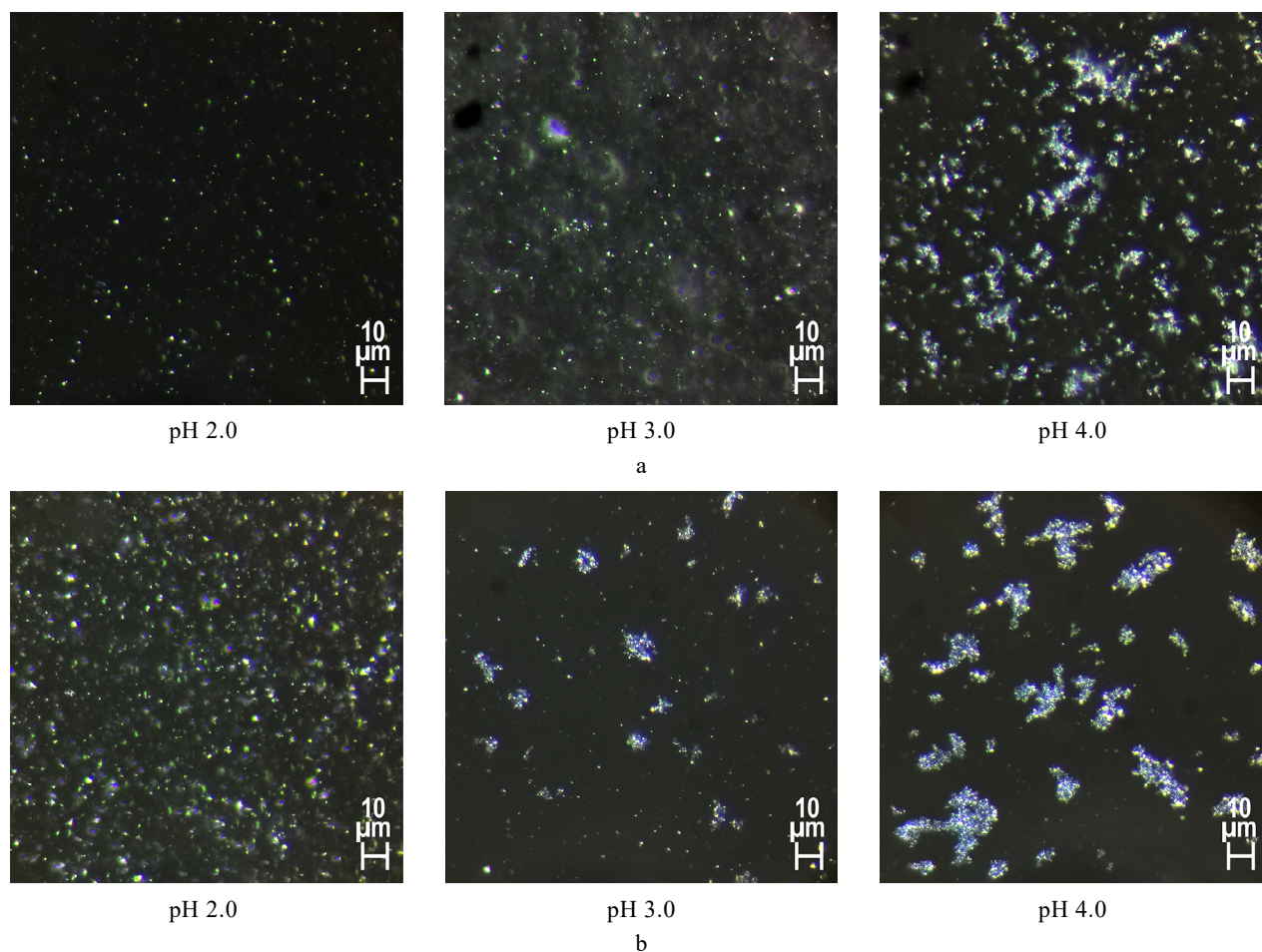


Figure 2. Protein substrates based on whey proteins before (a) and after (b) hydrolysis at 50°C

Рисунок 2. Микрофотографии белковых субстратов на основе сывороточных белков до (a) и после (b) гидролиза при температуре 50 °C

against whey proteins peaked at pH 2.9 for chicken pepsin and at pH 2.3 for the more acidophilic porcine pepsin.

We compared the curves showing the hydrolysis yield as a function of temperature and pH across different substrates. The plots for caseins and meat proteins were largely similar but differed significantly from those for whey proteins (Fig. 1). These differences were further corroborated by the F -values and the signs of factor effects derived from the analysis of variance (Table 1).

We also compared the F -values for the linear effects of temperature (Temp^L) and pH (pH^L). Varying the pH factor resulted in a greater change in the response variable (hydrolysis product yield) than varying the Temp factor. Thus, the hydrolysis yield depended more strongly on the pH level than on the hydrolysis temperature. Increasing the pH from 2.0 to 4.0 reduced the hydrolysis yield. Raising the temperature from 35 to 50°C increased the hydrolysis yield, as confirmed by the positive vector sign of the Temp^L effect. The linear effect values for temperature and pH significantly exceeded the quadratic effect values for these factors: the peptic hydrolysis of casein and meat proteins demonstrated an almost proportional

relationship between the hydrolysis product yield and the temperature and pH levels. The predominance of linear effects over quadratic effects for temperature and pH implied the absence of an extremum (maximum or minimum) for the response variable within the given temperature and pH ranges. Based on the signs of the linear effects (“+” for temperature and “–” for pH), the hydrolysis yield from casein and meat protein substrates increased, peaking when the temperature reached 50°C and the pH dropped down to 2.0.

Based on the statistically significant pairwise factor interaction effect, the impact of one factor on the variation of the response variable depended on the level of the other. Regarding the response variable (hydrolysis yield), the signs of the pairwise interaction effects between temperature and pH ($\text{Temp}^L \times \text{pH}^L$) differed depending on the type of substrate (casein or meat proteins). For casein, the sign of the $\text{Temp}^L \times \text{pH}^L$ interaction effect was positive, i.e., a higher temperature level resulted in a greater increase in hydrolysis at a higher pH level. For the meat protein substrate, the $\text{Temp}^L \times \text{pH}^L$ interaction effect had a negative sign: a lower pH level

avored a greater increase in the hydrolysis yield in response to rising temperatures.

The absolute value of the F -statistic for the $\text{Temp}^{\text{I}} \times \text{pH}^{\text{I}}$ interaction effect was substantially lower than that for the individual factors. Thus, the pairwise interaction does not have a noticeable effect on the degree of hydrolysis of casein and meat protein samples.

The magnitude and direction of the effects of temperature and pH on hydrolysis yield was similar for casein and meat protein substrates, unlike their effect on hydrolysis yield from whey proteins. This phenomenon could be attributed to structural differences across these substrates. Whey proteins were initially present in the substrate in their native form, with aggregation occurring as a result of hydrolysis. In contrast, proteins in casein and meat protein substrates were aggregated from the outset. Figure 3 shows micrographs of casein substrate preparations in buffer solutions at pH 2.0, 3.0, and 4.0.

Casein particles differ in size and shape in microscopic preparations. This variability could be attributed to the following factors. The pH of 4.6 is the isoelectric point of casein. It triggered the casein micelle aggregation, leading to gel formation. A further drop in pH below 4.0 intensified the forces that counteracted the aggregation. On one hand, the aggregation was promoted by hydrophobic interactions. These interactions were further enhanced by the rising temperatures [44], as well as by the aggregation activated by the hydrogen bonding, which resulted from the increased charge of amino acid side chains [36]. On the other hand, the increasing positive electrical charge of the casein molecules hindered the aggregation by generating electrostatic repulsive forces. The combined effect of these forces could lead to the development of dense casein gel. However, such external disruptive forces as vigorous stirring could lead to a suspension of fine particles instead of cohesive gel. Upon further acidification to pH values below 3.5, the solubility of casein particles increased due to the rise in positive charge and stronger electrostatic repulsive forces. At pH around 2.0, the casein gel redissolved, developing a stable solution [45].

L. Salelles *et al.* [5] prepared casein aggregate solutions by slow acidification: casein aggregates with a loose structure and porous surface formed within the pH range of 3.0–5.0. The authors reported no statistically significant differences in the sizes of aggregates formed at pH 3.0–5.0 (133–203 μm). The pH between 1.0 and 2.0 saw the development of dense aggregates with smooth surfaces. On average, the size of aggregates formed at pH 2.0 was half that of aggregates formed at pH 3.0–5.0. In our experiment, we prepared the substrate by stirring an aqueous casein solution with an acidic buffer solution, which prevented gel formation. As a result, the suspensions consisted of aggregates of varying sizes (Fig. 3).

The structure of casein curd was reported to affect the rate of pepsin hydrolysis. Casein hydrolysis is known to proceed much more slowly in dense curds than in loose,

porous ones [46]. A dense casein curd structure was reported to inhibit the hydrolysis rate because the diffusion of pepsin into the curd was hindered, meaning only the surface proteins underwent hydrolysis [47]. According to G. Somaratne *et al.* [48], the pepsin diffusion rate was higher in a looser protein gel network than in a denser gel. X. He *et al.* [49] reported that loosening the casein curd through high-pressure treatment (400–600 MPa) increased the rate of pepsin hydrolysis.

The pairwise interaction effect between temperature and pH (ANOVA, Table 1) could be attributed to the varying density of casein aggregates at different pH levels. This effect manifested as a tendency for the hydrolysis yield to decrease as the temperature rose from 35 to 50°C and the pH dropped from 4.0 to 2.0. The pre-hydrolysis microscopic examination of casein solutions revealed that the aggregates formed at pH 4.0 possessed a less dense structure and more irregular edges compared to those formed at pH 3.0 (Fig. 3). Consequently, the less dense aggregates produced at pH 4.0 were more susceptible to hydrolysis than the denser ones formed at pH 3.0. The tendency for hydrolysis to slow down when the pH dropped down to 2.0 could be attributed to other factors.

Peptides released during casein hydrolysis were able to self-assemble into aggregates, primarily through hydrophobic interactions and hydrogen bonding. The cores of these aggregates consisted of hydrophobic peptides, while amphiphilic peptides attached to these cores via hydrophobic regions and surrounded them. At pH 2.0, the hydrophobic interactions, as well as the enhanced hydrogen bonding and cation– π interactions, promoted the further association of hydrolyzed casein aggregates into an extensive three-dimensional network of macro-aggregates [47]. A similar branched network of aggregated protein particles was visible in the post-hydrolysis (pH 2.0) microscopic image of casein (Fig. 3).

The analysis of variance (Table 1), the dependence of hydrolysis yield on temperature and pH (Fig. 1), and the structure of protein particles in casein solutions (Fig. 3) made it possible to formulate a hypothesis to explain the slowdown in the casein hydrolysis yield as temperature rose at pH 2.0. The activity of chicken and porcine pepsins peaked under these conditions, while casein was virtually unaggregated and accessible for interaction with the enzymes (Fig. 3). As a result, rapid casein hydrolysis occurred, followed by the aggregation of released peptides. Some of the bonds susceptible to pepsin hydrolysis became buried within the aggregates and inaccessible to the enzyme, thus reducing the reaction rate and hydrolysis yield. This pattern was clear in the sample with chicken pepsin: as the pH dropped from 4.0 to 2.0, the increase in hydrolysis yield became progressively less pronounced (Fig. 1). For porcine pepsin, the sharp rise in activity at pH 2.0 accelerated the hydrolysis rate, compensating for the lower substrate accessibility and accelerating the yield (Fig. 1).

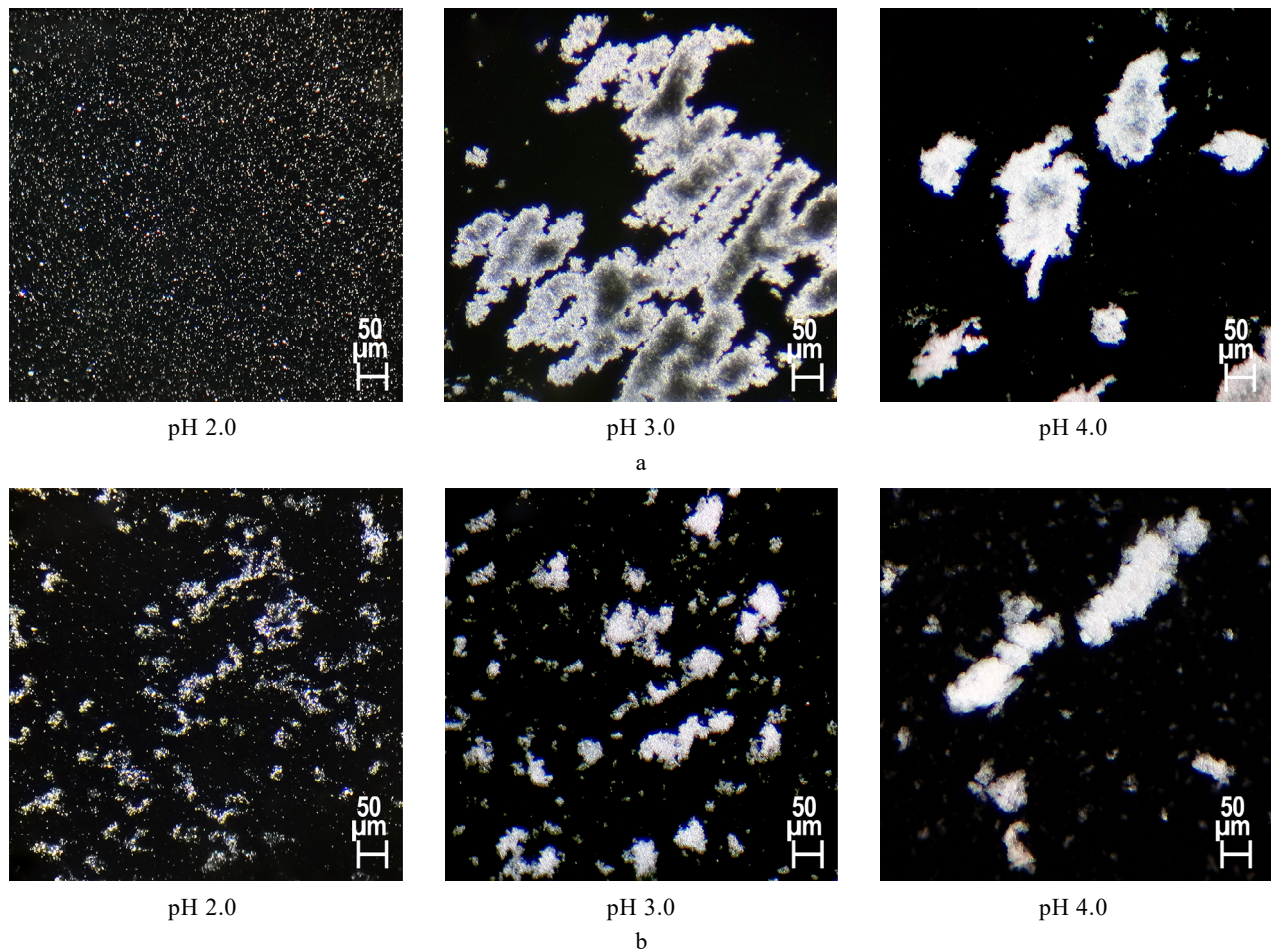


Figure 3. Protein substrates based on casein before (a) and after (b) hydrolysis at 50°C

Рисунок 3. Микрофотографии белковых субстратов на основе казеина до (a) и после (b) гидролиза при температуре 50 °C

Like the casein substrate, the meat protein substrate contained proteins in an aggregated form. Figure 4 shows micrographs of meat protein substrate preparations in buffer solutions at pH 2.0, 3.0, and 4.0.

These images were compared with electron microscopy images of collagen [48, 51, 52]. Based on the comparison, we were able to identify the large fibrous particles as collagen fibril fragments.

The meat protein substrate used in this experiment demonstrated a distinctive feature: a severe denaturation and aggregation of proteins. This feature was visible in the microscopic images, which revealed numerous insoluble particles. As with the casein substrate, the aggregation of meat proteins limited their accessibility to the enzyme, thereby reducing the hydrolysis rate.

The pre-hydrolysis pH of the substrate had no effect on protein particles and their accessibility to the enzyme. The hydrolytic activity of pepsin disrupted the structure of protein particles, enabling the enzyme to diffuse into the particles and further enhancing their hydrolysis [39]. At pH 4.0, we observed no hydrolysis-induced changes

in the structure of collagen particles. At pH 3.0–2.0, we detected hydration and structural breakdown of collagen fibrils. They disintegrated into smaller particles, which increased the substrate surface area available for contact with the enzyme. The extensive hydration improved the substrate solubility and further accelerated the hydrolysis. Similar changes occurred in other components of the meat protein substrate, including aggregates of myofibrillar and sarcoplasmic proteins. The sarcoplasmic proteins were so small that it was difficult to visualize any possible structural changes using light microscopy. However, the significant increase in the hydrolysis yield from the meat protein substrate as the pH dropped from 4.0 to 2.0 indicated their active cleavage by pepsin. The collagen hydrolysis products exhibited very low absorbance at 280 nm and could not contribute significantly to the hydrolysis yield, as measured by absorbance intensity at this wavelength. Therefore, the higher yield was attributed solely to the hydrolysis products from myofibrillar and sarcoplasmic proteins: they are rich in tyrosine and tryptophan, and their absorbance ability is high at 280 nm.

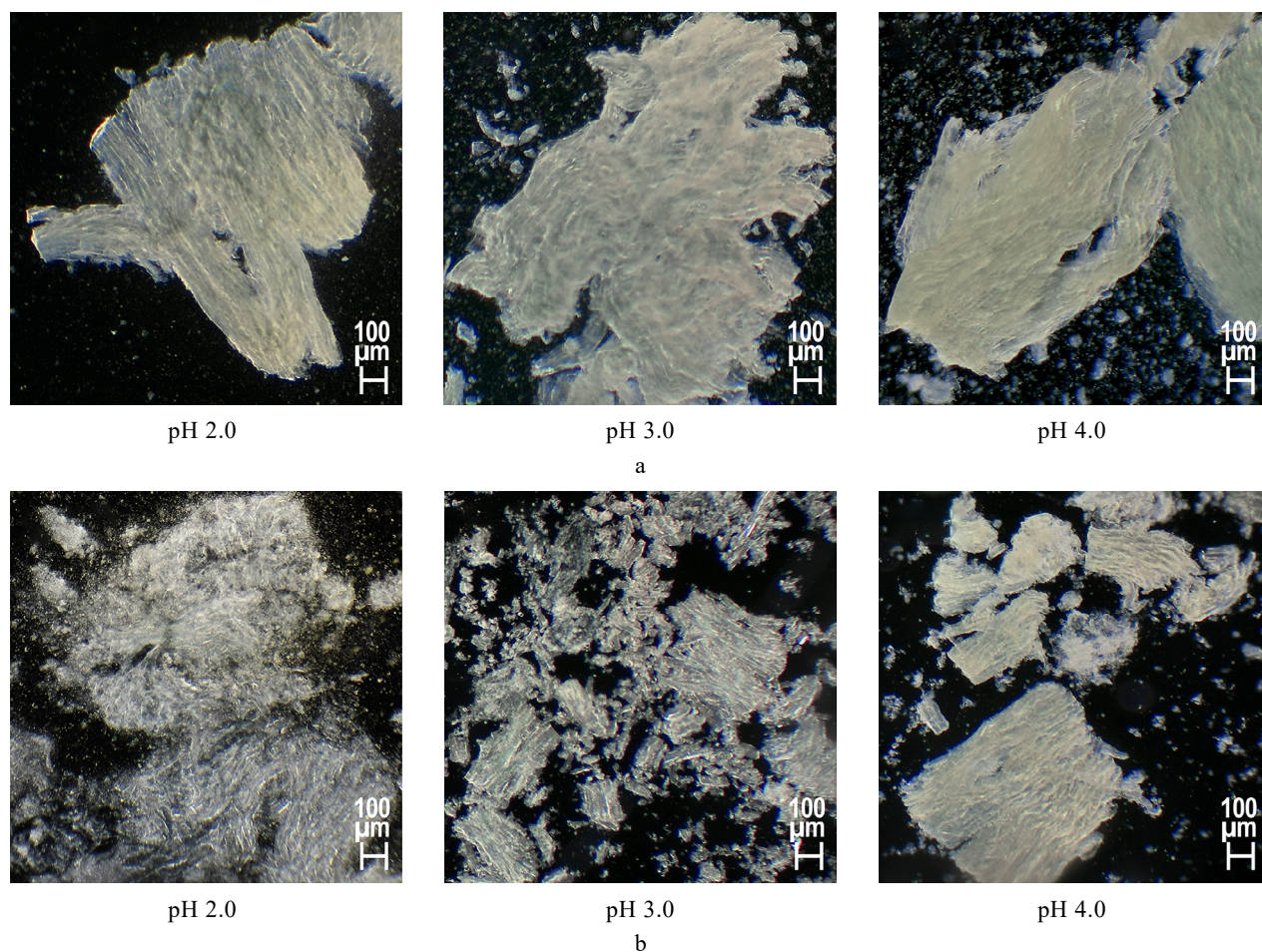


Figure 4. Protein substrates based on meat proteins before (a) and after (b) hydrolysis at 50°C

Рисунок 4. Микрофотографии белковых субстратов на основе мясных белков до (а) и после (б) гидролиза при температуре 50 °С

For casein, as the pH dropped to 2.0, the hydrolysis slowed down due to the formation of aggregates from the hydrolysis products and poor substrate accessibility. For meat proteins, the effect was the opposite: lowering the pH to 2.0 accelerated the hydrolysis due to the changes in the substrate structure and the exposure of numerous potential pepsin cleavage sites on the surface. This mechanism explains the negative vector sign of the $\text{Temp}^L \times \text{pH}^L$ interaction (ANOVA) in the model describing the hydrolysis product yield from meat proteins as a function of temperature and pH. Consequently, the hydrolysis yield from the meat protein substrate increased as the temperature went up and the pH went down.

Conclusions

For porcine and chicken pepsins, the highest hydrolysis yield had almost the same temperatures in the experiments with native whey proteins, caseins, and denatured meat proteins as substrates. Within the temperature range of 35–50°C, the highest yield occurred at 50°C. In fact, the temperature could have been higher, as pepsin is known to retain its activity up to 60°C. For a given pro-

tein substrate, chicken pepsin lost more activity than porcine pepsin as the temperature dropped from 50 to 35°C.

Within the pH range between 2.0 and 4.0, the highest yield from casein and meat protein substrates was observed at pH 2.0. In the experiment with native whey protein, it was at pH 2.9 for chicken pepsin and pH 2.3 for porcine pepsin. Porcine pepsin was more dependent on pH than chicken pepsin. Porcine pepsin activity dropped sharply as the pH rose from 2.0 to 3.0, even at the optimal hydrolysis temperature (50°C). The activity of chicken pepsin remained more stable under the same conditions.

Temperature and pH affected the hydrolysis rate not only by altering pepsin activity but also indirectly, by changing the substrate structure.

At pH 2.0, which was optimal for pepsin activity, β -lactoglobulin (a major whey protein) adopted a compact structure that hindered enzymatic hydrolysis. As the pH rose from 2.0 to 4.0, the susceptibility of β -lactoglobulin to hydrolysis increased, but the pepsin activity declined. A balance between these opposing processes was achieved within the pH range of 2.0–3.0,

where native whey protein substrates had the highest rate of peptic hydrolysis.

For caseins, lowering the pH from 4.0 to 3.0 accelerated the hydrolysis by increasing the pepsin activity and disrupting the casein aggregates. However, as the pH dropped down to 2.0, the hydrolysis slowed down due to the formation of secondary aggregates derived from casein hydrolysis products.

For meat proteins, lowering the pH from 4.0 to 2.0 accelerated the hydrolysis through the synergy of two mutually reinforcing processes: increased pepsin activity and greater substrate accessibility.

Based on these results, pepsins demonstrated strong potential for producing hydrolysates from meat proteins, particularly those highly denatured and aggregated after intensive heat treatment. In addition, the pepsin hydrolysis of meat substrates yielded products with high biological value and achieved effective protein bioconversion from the raw material.

Contribution

D.S. Myagkonosov supervised the research, wrote the review, analyzed the experimental results, and wrote

the manuscript. D.V. Abramov performed the experiments, processed the results, and wrote the manuscript. O.G. Kashnikova conducted the experiments, wrote the manuscript, and analyzed the results.

Conflict of interest

The authors declared no potential conflict of interest regarding the research, authorship, and/or publication of this article.

Критерии авторства

Д. С. Мяконосов – организация и руководство проведением исследований, анализ литературы и результатов экспериментов, написание статьи. Д. В. Абрамов – проведение экспериментов, обработка результатов, написание статьи. О. Г. Кашникова – проведение экспериментов, участие в написании статьи и анализе результатов.

Конфликт интересов

Авторы заявляют об отсутствии потенциальных конфликтов интересов в отношении исследования, авторства и / или публикации данной статьи.

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